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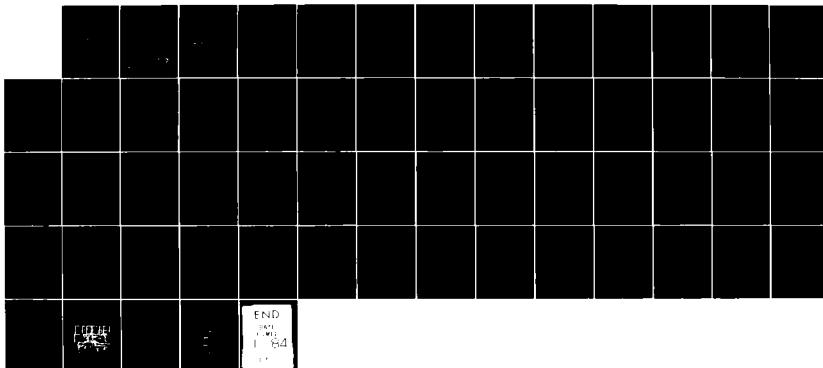
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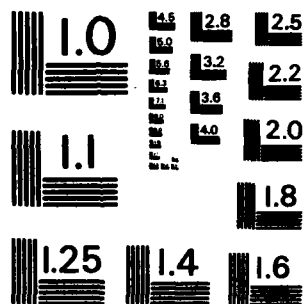
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ACTIVE SOLUTE TRANSPORT ACROSS PROG SKIN AND EPITHELIAL CELL SYSTEMS ACCORDING  
TO THE ASSOCIATION-INDUCTION HYPOTHESIS

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by

Gilbert N. Ling

SHORTENED TITLE: Active Transport of Bifacial Cells

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## 1. Introduction

The living cell is the basic physiologic unit of life. Some of the cardinal features of being alive may be revealed by studying the properties of a cell before and after it has ceased living. One method often used to tell a living from a dead cell is based on the dye-exclusion property. Dyes such as nigrosin, trypan blue, and erythrocin B are excluded by cells that are living but stain dead cells with vivid colors. The dead cell loses the ability to maintain itself separate from its environment.

The ability to exclude small molecules from living cells is not limited to foreign molecules like these dyes. It applies to normal components of the cell as well, only with a greater degree of subtlety, as in the case of the asymmetrical distribution of  $K^+$  and  $Na^+$  between cells and their environment. Chemically almost indistinguishable, this pair of ions is sharply segregated by living cells, which preferentially accumulate  $K^+$  to a level many times higher than that in the external medium and sustain a level of  $Na^+$  only a fraction of that found in the same external medium.

The earliest interpretation of this phenomenon of asymmetrical  $K^+$  and  $Na^+$  distribution of living cells was based on the assumption that the cell membrane was absolutely impermeable to both  $K^+$  and  $Na^+$  (1, 2). In years following revisions were made. First  $K^+$ , and later  $Na^+$ , were recognized to be permeant. After the 1940's, membrane impermeability as the basis of asymmetrical solute distribution was abandoned, largely in consequence of accurate and direct measurements of solute permeability made possible by the development of radioactive labelling and other techniques. There then remained two and only two possible interpretations for the asymmetrical solute distribution typified by  $K^+$  and  $Na^+$ : the steady state model in which the unequal distribution is due to continued operation of energy-consuming membrane pumps; and the equilibrium model in which the inside of the cell is considered different from that of the external medium and the accumulation

of  $K^+$  and exclusion of  $Na^+$  reflect the different physicochemical attributes of these different environments.

A strong argument often cited in favor of the steady state model is based on the ion-distribution pattern in giant algal cells such as *Volonia macrophysa*. Living in sea water, which contains nearly 50 times more  $Na^+$  than  $K^+$  (3), these cells may retain 3 times more  $K^+$  than  $Na^+$  (4). The bulk of  $K^+$  and  $Na^+$  in these algal cells is found in the cell sap contained in the central vacuole. Since the cell sap in fact contains little more than water and salt ions and is therefore not significantly different in this respect from sea water, clearly the maintained assymetry of  $K^+$  and  $Na^+$  distribution can only be due to active transport. This is a flawless argument but offers no proof that other non-algal cells accumulate  $K^+$  and exclude  $Na^+$  by the same mechanism. Indeed, giant algal cells with large central vacuoles are not typical of other living cells at all. Possession of a large central vacuole is only seen in old plant cells; young plant cells as well as most procaryotic and eucaryotic cells are "solid bodies" as pointed out by E. B. Wilson in his treatise, The Cell (5).

## 2. Concepts of Active Solute Transport Based on the Membrane-pump Theory

The majority of cell physiologists have adhered to the conventional membrane-pump theory. Studies of epithelial transport have been built on the basic tenets of the membrane-pump theory and hence epithelial solute transport and selective solute accumulation and exclusion in "simpler" cells have been considered equivalent, all due to membrane pumps. This approach has not taken full account of the difference between unifacial solid cells typified by human red blood cells, frog muscle and squid axon, and bifacial hollow cells typified by *Valonia*: (1) only *Valonia* contains an enclosed body of simple aqueous solution; and (2) only in *Valonia* is there a second membrane, the tonoplast, enclosing this internal aqueous phase. Fifty years ago, Chambers and Höfler (6) studied the osmotic behavior of the isolated central vacuole and showed that the tonoplast membrane has

quite different properties from the outer plasma membrane. Indeed the perfect osmometer-like behavior of plant cells was shown to be due primarily to the tonoplast and not to the plasma membrane.

Of course, giant algal cells are not the only living cell systems that conduct net transport of ions and other small molecules between two similar aqueous phases against concentration gradients. Many epithelial tissues do the same. Instead of the continuous protoplasmic layer of Valonia it is epithelial cells joined together in continuous sheets that separate the two simple aqueous phases. Without exception, each of these epithelial cells is bifacial, possessing two different surfaces facing each of the two aqueous media.

#### 2.1 The Proposed Mechanism of the Na Pump According to the Membrane-pump Theory.

As pointed out by Ussing and Leaf (7, p. 7), "It is the properties of the outward-facing membrane that are unusual, viz., the selectivity for sodium rather than potassium and the absence of a sodium pump." "The inward facing cell membrane..... can be assumed to be of the same nature as the sodium-potassium exchange pump of red cells, muscle and nerve". This opinion clearly shows that in the conventional view, epithelial  $\text{Na}^+$  transport is the same as in most unifacial non-epithelial cells. But what kind of mechanism, at least in theoretical terms, has been proposed for these Na pumps? The answer is clearly provided by Glynn and Karlish (8). Covering literature of a span of 23 years, these authors began their review on the sodium pump in these words, "The recent startling growth of the literature on the sodium pump may make a review timely... If the great mass of work that has been done had led to the general acceptance, even provisionally and even in outline, of a hypothesis accounting for the working of the pump we could have described that hypothesis and then considered the evidence for it. Unfortunately no such hypothesis exists....."

I believe that the major reasons behind the difficulty in proposing mechanism for the Na pump are faulty fundamental assumptions including the

concept of the lipid layer as the permeability barrier.

## 2.2 Theories of Epithelial Transport.

A number of theories have been offered relating to different aspects of epithelial transport. With the exception of the sketch of a theory communicated by myself in 1965 (see below) all were based on the membrane-pump theory.

### 2.2.1 "Two membrane Theory" of Koeber-Johnson and Ussing (9).

This theory was based on the authors' work with isolated frog skin. From electrical potential studies they reached the conclusion that the surface facing the outside solution has a selective high  $\text{Na}^+$  permeability and the surface facing the inside has a selective high  $\text{K}^+$  permeability. According to this well-known "two membrane" theory the inner membrane has essentially the same properties seen in most living cells including the possession of the Na-K pump in the form of  $\text{K}$ , Na-activated ATPase. It is the membrane facing the outside solution that is unusual and functions as the seat of transport regulation through the control of  $\text{Na}^+$  permeability.

### 2.2.2 The Standing Osmotic Gradient Theory of Diamond and Bossert (10).

Certain epithelial membranes like the gall bladder, intestinal mucosa, and renal proximal tubule transport salt ions and water in the form of an isotonic solution. Diamond and Bossert suggested that this secretion of an isotonic fluid is due to the pumping of ions into the bottom of the spaces formed between the folds of the baso-lateral membrane of the epithelial cells. The hypertonic solutions thus formed then draw water from the cell becoming more dilute and eventually isotonic as the fluid moves outward toward the serosal "sink".

### 2.2.3 Cereijido and Rotunno's Theory of Pericellular Pump (11)

In this theory Cereijido and coworkers argued that the transport of  $\text{Na}^+$  by frog skin involves the migration of  $\text{Na}^+$  along an array of fixed negative sites on the outside surface of the epithelial cells.

#### 2.2.4 The Na Gradient Hypothesis of Sugar and Amino Acid Transport

Based on the discovery that intestinal transport of D-glucose depends on the presence of  $\text{Na}^+$  in the mucosal fluid (12, 13), Crane (14), p. 439; 15) suggested that glucose,  $\text{Na}^+$  and a carrier form a ternary complex in the mucosal membrane which then dissociates and delivers  $\text{Na}^+$  and glucose. The  $\text{Na}^+$  gradient from the mucosal fluid to the cell interior provides the energy for the inward transport of sugar. That uptake of amino acids by unifacial duck erythrocytes and Ehrlich ascites cells and bifacial kidney cells require  $\text{Na}^+$  was known even earlier (16, 17). Schultz and Curran further elaborated the Na gradient hypothesis to include transport of amino acids (18). Support for the Na-gradient hypothesis came from the study of sugar and amino acid transport into isolated microvilli "vesicles". These isolated microvilli transiently take up more sugar or amino acids than in the surrounding medium, thereby exhibiting an "overshoot", when the sugar or amino acid is added with a high concentration of  $\text{Na}^+$  but not in the presence of sugar transport inhibitor, phloridzin. (references; see below)

#### 3. The Association-induction Hypothesis.

The bulk of cell  $\text{K}^+$  and  $\text{Na}^+$  in most living cells is not found in a separate aqueous phase, such as the central vacuole of Valonia, but is found in the cytoplasm where proteins make up from 15 to 25% of the total weight. According to the association-induction hypothesis (AI hypothesis) (19, 20), it is primarily the cell proteins which provide and maintain a different physico-chemical environment in the cell, and it is this different physico-chemical environment that gives rise to the accumulation of  $\text{K}^+$  and exclusion of  $\text{Na}^+$  (21). More specifically, the AI hypothesis argues that certain proteins provide a network or matrix of extended polypeptide chains whose alternating positive NH and negative CO groups polarize and orient the bulk of cell water in the state of polarized multilayers (22, 23). Water in this state has decreasing solubility for solutes with increasing molecular size and complexity. These same and/or other proteins provide

$\beta$ - and  $\gamma$ -carboxyl groups for the selective adsorption of  $K^+$  over  $Na^+$  (24).

However, both polarized water (and hence  $Na^+$  exclusion) and ion adsorption (and hence  $K^+$  accumulation) depend on the existence of the protein-water-ion system in a cooperative high energy state, called the living state. To maintain this living state, adsorption of ATP (and other key molecules) on certain controlling cardinal sites is essential (21).

Recently, there have been major developments in efforts aimed at choosing between the two diametrically opposed theories of the living cells: the steady-state membrane pump-leak theory, and the equilibrium AI hypothesis. Reserving a detailed discussion of this multifaceted, complex problem to a monograph in preparation and several more recent reviews (21, 25, 26), I shall very briefly discuss some of these problems in reference to the criticisms often cited against the AI hypothesis.

### 3.1 The Amount of Water of Hydration on Protein is Too Little to Support the Contention of the AI Hypothesis That The Bulk of Cell Water is Different From Normal Liquid Water (27)

Most native proteins hydrate to the extent of 0.2 to 0.3 gm water/gram of dry protein (23, 27). At this rate, no more than 7 to 8% of the cell water could be significantly affected by the 15 to 25% of proteins found in most living cells. However, recent surveys as well as experimental studies revealed that the above conclusion is valid only when proteins are in the globular conformation (23, 28, 29). On the other hand, if for one reason or another the protein exists in an extended conformation, long range polarisation of many more water molecules occurs. Water so affected has unusual properties, e.g., reduced solubility for  $Na^+$ , sugars, and amino acids, in full agreement with the AI hypothesis (29).

### 3.2 Strong Selective Adsorption of $K^+$ Over $Na^+$ Has Not Been Demonstrated in Isolated Proteins in Vitro (30).

Oubain-sensitive, cooperative selective  $K^+$  binding over  $Na^+$  onto one isolated protein (i.e., K, Na activated ATPase) has been successfully demonstrated

in vitro by Matsui and coworkers (31, 32). Even more exciting is the finding of Edelmann (33) who demonstrated selective accumulation of  $K^+$  over  $Na^+$  in frozen-dried muscle sections in vitro, a subject that will be dealt with in greater detail below.

3.3 If Cell  $K^+$ , Which Constitutes the Bulk of Intracellular Cation, is in a Adsorbed and Hence Osmotically Inactive Form, the Cell Would Not be Able to Maintain Its Normal Volume (34, 35).

The linear polymer, polyethylene oxide (PEO), which carries no net electric charge, polarizes water in deep layers. A dialysis bag containing PEO-water shrinks in concentrated Na-citrate solutions and swells in dilute Na-citrate solution in a manner similar to living cells when placed in hyper- or hypotonic solutions, even though in the case of PEO-water system there is no semipermeable membrane covering the polymer, the dialysis membrane being fully permeable to Na-citrate (36). This illustrates the point that in the AI hypothesis the reduction of the activity of cell water is due primarily to its interaction with macromolecules, not with  $K^+$ .

3.4 The Magnitude of the Cellular Resting Potential Demands That the Bulk of Cell  $K^+$  Must be in a Free State (37).

Cellular resting potential has been shown to be independent of intracellular  $K^+$  and external  $Cl^-$  concentration (19, 38, 39) thus contradicting the membrane potential theory of Bernstein (40, 41), the ionic theory of Hodgkin and Katz (42) as well as the electrogenic potential theory of Mullins and Noda (43, 44). On the other hand, the same data are in full quantitative agreement with the theory of the cell resting potential as a surface adsorption potential according to the AI hypothesis (19, 25, 38, 39, 45, 46).

3.5 Mobility of  $K^+$  in Squid Axon and Frog Muscles is Close to That of a Solute of Similar Ionic Strength with Some Slow-down Due to Mechanical Obstruction (47, 48).

$K^+$  mobility in healthy frog sartorius muscle fiber is only 1/8 of that in a solution of 0.1 M KCl (49). Deterioration of the cells causes increases of mobility approaching that of a dilute salt solution.

3.5 Using Ion-selective Intracellular Electrodes, the  $K^+$  Activity in Nerve and Muscle Cells is Close to That of a Simple KCl Solution of Equal Ionic Strength (50, 51).

The question of intracellular  $K^+$  activity is the subject of the next section.

4. The Physical State of  $K^+$  in Living Cells.

4.1 Evidence for  $K^+$  Adsorption.

Khuri (52) wrote in a recent review, "Since intracellular activities of the monovalent ions were determined largely in muscle fibers... but no activity values were available for epithelia, renal physiologists generally assumed that as in muscle, intracellular  $K^+$  is all free... As it turned out, these extrapolations with respect to the physical state of intracellular K... were invalidated by the intracellular electrochemical techniques." Table 1 compiles data on the intracellular  $K^+$  activities measured in a variety of epithelial cells. Uniformly, the activity coefficient measured was markedly below that found in a solute solution. Most workers explained the reduced  $K^+$  activity as due either to adsorption of  $K^+$  in the epithelial cells or to sequestration of the missing free  $K^+$  in some subcellular compartments. The second explanation can be shown to be highly unlikely.  $K^+$  is by far the major cation in the cells; most  $Ca^{++}$  and  $Mg^+$  are bound;  $Na^+$ -sensitive microelectrode studies showed that there is also very little free  $Na^+$  activity in the epithelial cells (ca. 20 to 25 mM) (52, p. 71). With little other osmotically active solutes to balance the deficit, sequestration of the missing  $K^+$  into the limited volume of organelles, which make up less than 50% of

cell volume, would lead to an impermissible osmotic imbalance.\* This difficulty leaves only  $K^+$  adsorption as the more reasonable explanation.

The question must be raised next, Is  $K^+$  all "free" in muscle cells, as Khuri mentioned? A possible answer to this question was offered some time ago (49, 53): protoplasm is easily damaged or activated by the intrusion of the ion-sensitive electrode which excites and causes liberation of  $K^+$  from its adsorbing sites. The fact that the rest of the cytoplasm distant from the microelectrode may remain intact is irrelevant, because it is only the  $K^+$  activity in the immediate vicinity of the microelectrode tip that is measured. The lower  $K^+$  activity recorded in epithelial cells suggests that they are less susceptible to activation by the microelectrode than muscle and nerve cells which are inherently more excitable.

The question of the state of  $K^+$  in muscle cells has been vastly clarified mostly through the brilliant work of the young German scientist, Ludwig Edelman.

According to the AI hypothesis, the seats of selective  $K^+$  adsorption are the  $\beta$ - and  $\gamma$ -carboxyl groups of certain intracellular proteins (19, 24, 53, 54). In muscle cells, more than 60% of the  $\beta$ - and  $\gamma$ -carboxyl groups belong to myosin (54). Since in striated muscle, myosin constitutes the A bands, one could expect  $K^+$  to be localized primarily in these bands. Furthermore, since it is generally accepted that in EM sections, it is also  $\beta$ - and  $\gamma$ -carboxyl groups that bind and are thus stained by cationic uranyl and lead stains, the AI hypothesis could further predict that in normal living striated muscle (as well as in other cells)  $K^+$

\* The total intracellular  $K^+$  concentration is approximately 100 mM. Let us make the generous assumption that the combined volume of mitochondria, nuclei and other subcellular particles add up to 50% of the cell volume. The measured  $K^+$  activity is 40 mM. To explain this low  $K^+$  activity in terms of sequestration the average  $K^+$  concentration in the subcellular particles would be  $100 \div (100 - 40) = 160$  mM. The sum of measured cytosol  $K^+$  and  $Na^+$  would then be  $40 + 25 = 65$  mM, or less than one half of the anticipated subcellular particle osmotic activity and thus untenable.

will be distributed at the same sites that appear dark in standard fixed EM sections stained with uranium and lead.

Figure 1 assembled from the published work of Edelmann (55) shows that these expectations are indeed true. Figure A is a reproduction of a small segment of frog muscle fixed and stained with uranium and lead the usual way. Figures B and C, are similar muscle sections which had not been fixed or chemically stained. Instead the muscles were first loaded with electron-dense  $\text{Cs}^+$  (A) or thallium ( $\text{Tl}^+$ ) (B and C) by prior incubation in Ringer solutions whose  $\text{K}^+$  had been replaced by  $\text{Cs}^+$  or  $\text{Tl}^+$ . These  $\text{Cs}^+$  or  $\text{Tl}^+$ -loaded muscles were then freeze-dried, infiltrated with Spurr's medium and dry-cut with the simple and efficient technique of Edelmann (56). Figure D shows a cesium-loaded section after it had been washed in distilled water. Figure E shows normal frog muscle with its normal  $\text{K}^+$ -content. Figure 1 confirms the expectation that  $\text{K}^+$  in muscle cells is neither free nor evenly distributed but is adsorbed on  $\beta$ - and  $\gamma$ -carboxyl groups belonging to the A band and Z-lines.

The validity of these conclusions was further confirmed by three different laboratories using two additional types of techniques: (i) radioautography of  $\text{Cs}^{134}$  and  $\text{Tl}^{208}$ -loaded, air-dried single muscle fibers (57); (ii) low temperature ( $70^\circ \text{K}$ ) radioautography of  $\text{Cs}^{134}$ -loaded, frozen single muscle fibers (58); and (iii) dispersive x-ray microprobe analysis of  $\text{Cs}^+$  and  $\text{Tl}^+$ -loaded muscle and of muscle with its normal  $\text{K}^+$  contents (59). The latter was also confirmed by Trombitas and Tigyi-Sebes in isolated air-dried single myofibril of honeybee thorax muscle (60).

There is no doubt that virtually all of the  $\text{K}^+$  is specifically adsorbed onto specialized locations in the striated muscle cells. Other experiments both in intact muscle cells (54) and in muscle cells whose postulated membrane pumps had been made totally non-functional (EMOC preparation) showed clearly that the  $\text{K}^+$  is specifically adsorbed onto the anionic sites and not merely hovering around as

free counterions (61, 62): In displacing  $K^+$  from adsorption, a pair of univalent cations (e.g.,  $K^+$  vs.  $Cs^+$ ,  $Cs^+$  vs.  $Tl^+$ ) have quantitatively different effects indicating the major role played by short range attributes which alone distinguish these ions and which cannot be "felt" without direct contact between the anionic sites and the adsorbed ions (57).

These findings reinforce the earlier suggestion that ionic activities measured with intracellular ion-sensitive electrodes must be regarded with great caution not only in excitable cells like muscle and nerve but even in epithelial and other cells. The  $K^+$  activity coefficient measured with an intracellular electrode tends to be higher than it should be in totally undisturbed cells. According to the AI Hypothesis, virtually all  $K^+$  in living cells is in the adsorbed state (Fig. 1).

#### 4.2 The Implications of the Establishment of the Adsorbed State of the Bulk of Intracellular $K^+$ .

##### 4.2.1 Osmotic Balance.

The immobilization of the bulk of intracellular  $K^+$  leaves a major osmotic deficit in muscle cells. One recalls that it is precisely this anticipated "difficulty" that one time led Hill and many others to conclude that cell  $K^+$  must be all free. Since  $Ca^{++}$  and  $Mg^{++}$  as well as a substantial portion of  $Na^+$  in frog muscle cells are also in an adsorbed and hence osmotically inactive state, there is no other recourse but to accept that the osmotic activity, that is the lowering of activity of water, must arise from cell proteins. This agrees well with the polarized multilayer theory of cell water in which water activity is reduced by long range polarization and adsorption. In other words, osmotically speaking, "pure" polarized water behaves like normal liquid water in which substantial amounts of ions or other solutes are dissolved.

##### 4.2.2 Cellular Electric Potential.

The conventional view of the cellular electrical potential as first suggested by Ostwald (63) and extensively developed by Bernstein (40) as

well as later variants introduced by Boyle and Conway (41), by Hodgkin and Katz (42), and Mullins and Noda (43) were all based on the assumption that the bulk of intracellular  $K^+$  is in the free state. That in fact the bulk of intracellular  $K^+$  is in a localized, adsorbed state made all these theories unapplicable. Again, this adsorbed state of cell  $K^+$  is perfectly in harmony with the AI Hypothesis' surface adsorption theory of cellular resting potential, which has already been experimentally tested and verified (38, 46).

##### 5. The Nature of the Surface Barrier of Living Cells and Subcellular Particles.

Since Overton introduced his lipoidal membrane theory (64) the idea that all cells as well as subcellular particles are covered by a membrane whose continuous phase is lipid in nature has sunken deep roots. Yet there had long been serious questions about the validity of this fundamental assumption (65, 66). Seven years ago I presented evidence in support of the view that not lipid, but a layer of water polarized in multilayers by surface proteins, constitutes the semipermeable surface barrier (65).

Recent work of Stillman et al on squid axon (67), of Maloff et al on inner mitochondrial membrane (68), and of Ling and Ochsenfeld on frog muscle and egg (69) offer additional experimental evidence: No change of  $K^+$  permeability was observed in any of these systems studied in response to  $K^+$  ionophores. Since there is no question that under similar conditions  $K^+$  permeability of phospholipid layers is drastically increased by valinomycin ( $10^{-7}$  M) and monactin ( $10^{-5}$  M) (70) one concludes that the semipermeable barrier in muscle, nerve, and egg cells as well as the inner membrane of mitochondria, is not a phospholipid layer.

In my view of the cell surface barrier, semipermeability depends on the state of multilayer polarization of cell water near the surface. The state of multilayer polarization of cell surface water in turn depends on certain proteins assuming the extended conformation. Semipermeability is therefore under close

control by hormones or drugs that react with receptor sites on these proteins and so control their conformation. The susceptibility of water to allosteric control, offers yet another major advantage to the association-induction model and hopefully new approaches to cell physiology and pharmacology.

#### 6. Solute Permeation According to the AI Hypothesis.

Before a full presentation of the theoretical model of active transport of solute and water across bifacial cells can be given, some additional basic concepts of the AI Hypothesis need to be introduced. These concepts include (i) the molecular mechanism of selective solute distribution in living cells, (ii) the molecular mechanism of selective solute permeability, (iii) cooperativity in adsorption and desorption of solutes and water, and (iv) the control of cooperative adsorption - desorption by the cardinal adsorbent, ATP. Fuller accounts of these concepts and supportive evidence can be found in reviews cited (19, 20, 21, 26, 71).

##### 6.1 Selective Solute Distribution in Living Cells

There are two basic modes of existence of an intracellular solute: adsorbed on macromolecular sites, or dissolved in the cell water. Referring to the particular solute of interest as  $p_i$ , and its concentration in the cell in moles per kilogram of fresh cells as  $[p_i]_{in}$ , then

$$[p_i]_{in} = [p_i]_{free} + [p_i]_{ad} , \quad (1)$$

where  $[p_i]_{free}$  and  $[p_i]_{ad}$  are respectively the concentration of free and adsorbed  $i$ th solutes in moles per kilogram of fresh cells. The distribution of free  $i$ th solute in the cell water follows the Nernst distribution law and is thus described by the linear relation

$$[p_i]_{free} = \alpha_i [p_i]_{ex} . \quad (2)$$

where  $\alpha$  is the percentage water content (v/w) of the cell.  $[p_i]_{\text{ex}}$  is the equilibration concentration of the  $i$ th solute in the external medium.  $q_i$  is the average equilibrium distribution coefficient of the  $i$ th solute between cell water and the external medium. Now, if and when the cell water is entirely normal as in, say a Ringer solution,  $q_i$  would be equal to unity for all permeant solutes. If, however, the cell is in its normal resting state, then according to the AI Hypothesis, this water is not normal liquid water but in the state of polarized multilayers. As a rule water in the state of polarized multilayers has different solubilities than normal liquid water, and in polarized water  $q_i$  varies with the solute involved. In general, small and simple molecules have  $q$ -values close to or equal to 1. The  $q$ -value usually decreases with increasing size and complexity of the solute in question. The concentration of hydrated solutes like  $\text{Na}^+$  in the living cell may be 0.1 or even lower. According to the AI Hypothesis, it is the low  $q$ -value for  $\text{Na}^+$  in the cell water and the unfavorable adsorption energy of  $\beta$ - and  $\gamma$ -carboxyl groups in comparison to that for  $\text{K}^+$ , that account for the sustained low  $\text{Na}^+$  concentration in many cells.

The adsorbed fraction of the  $i$ th solute may be, under the simplest condition, limited to just one type of adsorption sites. In this case, the concentration of the  $i$ th adsorbed solute may be described by a Langmuir adsorption isotherm:

$$[p_i]_{\text{ad}} = \frac{[f] K_1 [p_i]_{\text{ex}}}{1 + K_1 [p_i]_{\text{ex}}}, \quad (3)$$

where  $[f]$  is the concentration of intracellular adsorption sites in moles per kilogram of fresh cells.  $K_1$  is the adsorption constant in units of  $(\text{M})^{-1}$ . If another solute called the  $j$ th, and represented as  $p_j$ , also adsorbs onto the same sites, Equation 3 becomes

$$[p_i]_{ad} = \frac{[f] K_i [p_i]_{ex}}{1 + K_i [p_i]_{ex} + K_j [p_j]_{ex}} \quad (4)$$

Equation 4 shows that there is a hyperbolic relation between the adsorbed *i*th solute and the external concentration of the *i*th solute. Further, this fraction shows competition with other solutes like the *j*th and saturability, i.e., as  $[p_i]_{ex}$  increases,  $[p_i]_{ad}$  approaches but cannot exceed the value of  $[f]$ .

Combining Equations 1, 2 and 4, we have

$$[p_i]_{in} = \alpha q_i [p_i]_{ex} + \frac{[f] K_i [p_i]_{ex}}{1 + K_i [p_i]_{ex} + K_j [p_j]_{ex}} \quad (5)$$

A somewhat simpler version of this equation was first presented by the Russian physiologist, A. S. Troschin (72).

## 6.2 Selective Solute Permeability

### 6.2.1 Selective Solute Permeation in Terms of the Membrane-pump Theory.

In the conventional membrane theory, permeation of a solute molecule into a living cell may be achieved by three mechanisms: (i) free diffusion, (ii) facilitated diffusion, and (iii) active transport.

Free diffusion is, as the name indicates, envisaged as involving diffusion through the lipid phase of the cell membrane. The rate of a solute entering into a cell by free diffusion is usually linearly related to the concentration of the solute in the external medium. Facilitated diffusion and active transport mechanisms are fundamentally alike. For example, both show saturability. Their difference lies in the relative electrochemical potential of the phase the solute moves into. An active transport mechanism of solute movement is conceived as being against an electro-chemical gradient, while facilitated diffusion is conceived as being not against an electrochemical gradient. The notion of an uphill

movement when applied to unifacial cells is entirely dependant on the basic assumption of the membrane theory that the cell interior is filled with a dilute aqueous solution. Since this assumption has already been disproven (see above), there is no longer a solid foundation for this difference between facilitated diffusion and active transport into single unifacial resting cells such as erythrocytes, muscle or nerve cells. Thus phenomenologically there are two rather than three types of permeation mechanisms: one is saturable; the other is not.

Facilitated diffusion is distinguished from free diffusion by at least three outstanding features: (i) Saturability. As already mentioned, the rate of entry of a solute into living cells by this means does not increase linearly with increasing external concentration of the solute. Instead, the rate levels off to approach a fixed value. (ii) Competition: The rate of entry of a solute into living cells is inhibited by other solutes of similar nature. In practice, this trait can be easily and quantitatively assessed with the aid of, for example, Lineweaver and Burk double reciprocal plots of rates vs. external concentration, as in enzyme kinetic studies. (iii) Specificity: The rate of entry of a solute into living cells often exhibits a high degree of specificity. Thus the steric orientation of one of the 5 OH groups in a sugar molecule may have a profound influence on the rate of entry of the sugar. It has been widely considered that facilitated diffusion is mediated through "molecular carriers", which like ferry boats select favored "passengers" and shuttle them back and forth across the lipid membrane barrier. We have already mentioned the evidence against the concept that cells are covered with a continuous sheet of phospholipid. Other evidence against the lipid barrier concept has been reviewed elsewhere (67 - 69). The disproof of the universal existence of a continuous layer of lipids, also has removed the foundation of the concept of "mobile carriers", which like ferry boats, cannot shuttle and perform their transport function without a fluid barrier. Indeed, from the viewpoint of the conventional membrane theory, one could not have imagined a better "mobile carrier" for  $K^+$  than valinomycin and monactin. The failure of these

"ionophores" to cause any significant change in  $K^+$  permeability in nerve, muscle, and egg cells demands totally different interpretations for the physiological observations of solute permeation. Fortunately, such an alternative view has been available for quite some time already (19, 39, 73, 74).

#### 6.2.2 The Mechanism of Solute Permeation According to the AI Hypothesis

Proteins, lipids and water are the three major components of all living matter. Since lipid does not constitute a continuous surface barrier and a "mobile carrier" cannot exist without a water-immissible fluid barrier, clearly a different phospholipid model must be sought to explain the competitive and saturable solute permeation widely observed. Indeed such a model was suggested

and will now be briefly reviewed (19, 75).

##### 6.2.2.1 Polarized Water in lieu of Lipid Layer as the Surface Barrier for "Non-saturable" Solute Entry.

The near perfect semipermeability of Traube's copper ferrocyanide precipitation membrane led Pfeffer to found the membrane theory (1). Copper ferrocyanide obviously contains no lipid but is a gel containing a network of crystalline particles with water-filled interstices on the order of  $150 \text{ \AA}$  in width (76, p. 656). These "pores" are many times as wide as the diameter of sucrose (diameter  $94 \text{ \AA}$ ); yet, this membrane is virtually impermeable to sucrose. More recently I have shown that the permeability to water and 10 other hydroxylic compounds at 3 different temperatures through inverted frog skin shows excellent correspondence to that through a sheet of cellulose acetate (65). Both membranes exhibit highest permeability for water; both were virtually impermeable to sucrose even though the average pore diameter of the "active" layer of the cellulose acetate membrane is more than 4 times as wide as the sucrose molecule. These data offer strong support for the concept that water in the state of multilayers, polarized by a matrix of protein chains carrying polar sites, provides semipermeable surface barriers in living cells as well as their cogent models.

6.2.2.2 Fixed Polar Sites on Side-chains and "Backbone" of Cell Surface Proteins in lieu of "Mobile Carriers" and "Pumps" as the "Gates" for "Saturable" Solute Entry.

The AI Hypothesis offers an interpretation of what is conventionally called facilitated diffusion, as well as of active transport, in terms of an adsorption-desorption mechanism on cell surface adsorption sites. Indeed the mechanism is so simple that it involves few additional postulations than those already described for solute distribution in the bulk phase.

Consider the rate of permeation of labelled  $K^+$  into a living cell, typically demonstrating saturability, competition and a high degree of specificity. Now the bulk phase distribution of  $K^+$  in frog muscle cells is to first approximation described by Equation 5. If we consider the cell surface to be also primarily a water-protein system like the cell interior, then in principle the cell surface is a two dimensional rendition of the three-dimensional cell. In other words, like the cell interior, the cell surface will have a continuous layer of polarized water mentioned above, and also anionic  $\beta$ - and  $\gamma$ -carboxyl groups which are distributed at regular distances apart. Furthermore, if an instantaneous photograph could be taken, one would find most of the  $K^+$  to be associated with the surface anionic sites, as has been demonstrated for the bulk phase  $K^+$  (Figure 1). Few  $K^+$  molecules would be found between these sites in the interstices filled with polarized water. If now a motion picture is taken, one may observe two different modes of entry corresponding to each of these instantaneous positions taken by the  $K^+$ .

- (1) Saltatory route. In this mode of entry,  $K^+$  enters via the polarized water filling the space away from the charged sites.
- (2) Adsorption-desorption route. As the name indicates the  $K^+$  from the outside must first successfully occupy one of the anionic sites, followed by a librational

motion around the anionic site and then eventually by its desorption and entry into the cell.

Taking both routes into account, the rate of entry  $v_1^{inw}$  of the  $i$ th ion in moles per sec. per kilogram of fresh cells can then be written as (19, 74)

$$v_1^{inw} = Ak_{sal}^i [p_i]_{ex} + \frac{k_{Ad}^i [f] K_i [p_i]_{ex}}{1 + K_i [p_i]_{ex} + K_j [p_j]_{ex}} \quad (6)$$

where  $A$  is the total surface area of one kilogram of cells,  $k_{sal}^i$  is the inward rate constant for the entry of the  $i$ th solute via the saltatory route.  $[f]$  is the molar concentration of surface sites per kilogram of fresh cells.  $k_{sal}^i$  is a kinetic rate constant for the desorption of the  $i$ th solute from the surface adsorption sites.  $K_i$  and  $K_j$  are respectively the adsorption constants in  $M^{-1}$  of the  $i$ th and  $j$ th solute on the surface sites.

Epstein and Hagen (77) in 1952 first successfully analyzed alkali-metal ion entry into barley roots using Michaelis-Menton Kinetics, a finding I soon confirmed for  $Rb^+$  entry into frog muscle (73). Epstein and Hagen, and many other investigators who have extended these studies to other types of cells and solutes, adhered to the "mobile carrier" concept. Indeed with the additional assignment of the non-saturable fraction of solute entry as "leak", the overall rate equation for solute permeation becomes formally analogous to Equation 6. (for extensive discussion of this subject from the conventional viewpoint, see Christensen, 78). However, as mentioned above, the disproof of the lipid layer theory makes untenable the mobile carrier model.

To demonstrate the general validity of Equation 6 and its basic assumptions, we have demonstrated that Equation 6 can not only describe the rate of entry of solutes into living cells but also entry into such inanimate systems:

as sheets of ion exchange resin and sheep's wool (19, 39, 74). These models share the common attributes of the AI model of anionic sites fixed in a matrix of polarized water.

While Equation 6 adequately describes solute entry especially for solutes that are neutral, a somewhat more complicated equation is required to describe the entry of ions into frog muscle cells by a mechanism called the triplet adsorption-desorption route. That is to say, in the case where the entrant ion is rather tightly adsorbed by the electrostatic forces, its desorption requires the participation of another free cation. Thus a second  $K^+$  from the outside may approach a fixed anion -  $K^+$  pair in the right direction, weakening the attraction between the fixed anion and  $K^+$ , thereby facilitating the latter's entry into the cell. Indeed, we have shown that the rate of entry of labelled  $Rb^+$  into frog muscle cells is facilitated by external  $K^+$  (74). Clearly the facilitating effect of the second  $K^+$  outweighs the competition offered by  $K^+$  against  $Rb^+$  adsorption.

#### 6.2.2.3 Specificity of Sugar Permeability

The third characteristic of permeation phenomena is specificity. This has often in the past been attributed to carriers, and is exemplified by sugar entry into cells. It is here, I believe that the present model also has distinctive advantages over the carrier model.

First there is little question that the high degree of steric specificity for solute permeation rate can only be recognized by a system that provides a complex of closely spaced sites. This strongly suggests that these sites are provided by proteins. In the AI model, adsorption followed by libration and desorption would be all that is required to achieve a facilitated diffusion when such specific sites are available.

#### 6.2.2.4 Specificity in Alkali-metal Ion Entry; Its Variability and the c-value Concept.

Surfaces of most normal uniaxial cells and the basal lateral surfaces of biaxial cells have greater permeability for  $K^+$  than for  $Na^+$ . However, the frog skin surface facing the outside has a higher permeability for  $Na^+$  than for  $K^+$  ((9). What could be the molecular basis for this specificity difference?

In 1952 I suggested a theoretical model of selective adsorption of  $K^+$  over  $Na^+$  on fixed anionic sites (53). Stimulated by the later discovery that carboxyl ion exchange resin selects  $Na^+$  over  $K^+$  (79) while sulfonate ion exchange resin selects  $K^+$  over  $Na^+$ , as well as the work of Eisenman, Rudin and Casby on glass electrode ion selectivity, I constructed a theoretical model in which the c-value concept was introduced (19, 39). In essence, the c-value measures the electron density of an anionic oxygen atom; high c-value is equivalent to a high pK value as in acetic acid; a low c-value to a low pK value as in trichloroacetic acid. It was then possible to show that a variation of the c-value produces changes in the preferential order of selectivity among  $Cs^+$ ,  $Rb^+$ ,  $K^+$ ,  $Na^+$ ,  $Li^+$ , as well as  $NH_4^+$  and  $H^+$ . Thus  $K^+/Na^+$  preferences seen in muscle and nerve as well as basolateral membranes of various epithelial cells corresponds to a fairly low c-value; high  $Na^+$  over  $K^+$  preference seen in a number of apical cell membranes (9) bespeaks of a high c-value. The essence of this work was presented in 1960 (39) and in full detail in 1962 (19).

Solitary  $\beta$ - and  $\gamma$ -carboxyl groups usually have a pH value of 4 to 5; when carboxyl groups are placed in close proximity to each other as in various carboxy types of ion exchange resin, the pK value may rise to 9 or even higher (80). Thus the high c-value of apical membranes may be due to  $\beta$ - and  $\gamma$ -carboxyl groups in close proximity to each other or in pairs, while the low c-value at the basolateral membrane may be due to isolated  $\beta$ - and  $\gamma$ -carboxyl groups (for evidence, see 81).

In 1962 Eisenman also published a theory of selective ionic adsorption using a much simpler model (82, see also 83) to explain the

relation between ion specificity of glass electrodes and the glass composition (83, 84). Ussing and Leaf (67) considered Eisenman's theory to explain the different specificity at the two surfaces of epithelia but rejected it on the ground that the theory does not provide enough specificity. Instead, they preferred the "close-fit" hypothesis of Mullins (85, 86) in which closefitting into small holes endows a high degree of specificity for selectivity for  $\text{Na}^+$  and  $\text{Li}^+$  over  $\text{K}^+$  (see 19, p. 548, for tabulated data from literature). Mullins' model required dehydration of  $\text{Na}^+$  and  $\text{Li}^+$  prior to entry into the postulated close-fitting pores - a concept that is not easily defensible even if the cell membrane were a lipid bilayer. The disproof of the lipid bilayer membrane theory has left the close-fitting pore concept without a foundation. It is even more difficult to think of rigid pores in a layer of polarized water that would force the dehydration of  $\text{Na}^+$  and  $\text{Li}^+$  before their entry.

On the other hand, the low level of ionic specificity attributed to Eisenman's model is not applicable to the association-induction model in which a high degree of  $\text{Na}^+$  as well as  $\text{K}^+$  selectivity has been theoretically calculated.

In brief, I feel that difference in ionic specificity of the two surfaces of the epithelial cells can be adequately explained as a result of the difference in the c-values of the anionic surface sites.

## 7. The Cooperative Protein-Water-Ion Assembly.

### 7.1 Cooperativity in Adsorption and Desorption of Solutes and Water.

In the preceding sections we have discussed adsorption in relation to selective solute accumulation as well as selective solute permeability. Thus far, these adsorption sites were considered as non-interacting and therefore the adsorption is adequately described by the Langmuir adsorption isotherm. In a Langmuir adsorption isotherm as shown as a part of Equation 5, the concentration of adsorption sites  $[f]$  and a pair of adsorption constants  $K_1$  and  $K_2$  (or their ratio

$K_1/K_2$ ) determines the adsorption at a fixed ratio of  $[p_1]_{\text{ex}}/[p_2]_{\text{ex}}$  in the surrounding medium. In the cooperative adsorption isotherm, these parameters play comparable roles. However, it is the new parameter, the nearest neighbor interaction energy, that opens the door toward coherence in adsorption. Thus if the nearest neighbor interaction energy is positive, it means that if one adsorption site adsorbs  $K^+$ , it would make the two immediately neighboring sites prefer  $K^+$  over  $Na^+$ . Conversely if the middle site adsorbs  $Na^+$ , it would make the two immediately neighboring sites prefer  $Na^+$  more than if the middle site adsorbs  $K^+$ . The result is autocooperativity in ion adsorption. When the nearest neighbor interaction energy is large and positive, the whole system of adsorption sites will either adsorb all  $K^+$  or all  $Na^+$ . "All or none" switching can then occur at a "threshold" value of external  $K^+/Na^+$  concentration ratio. Thus autocooperativity among the 4 heme sites in hemoglobin provides the molecular basis for efficient loading and unloading of oxygen between the lung and the respiring tissues. This type of cooperative adsorption isotherm also describes the uptake of  $K^+$  or  $Na^+$  in various living cells including frog muscle (87, 88, 89), human lymphocytes (90, 91) and a variety of smooth muscles (92, p. 22) all showing positive nearest neighbor interaction and a sigmoid-shaped adsorption curve. Since we have now established that initially nearly all intracellular  $K^+$  is in an adsorbed state (Figure 1), this type of autocooperative switching from the  $K^+$  to the  $Na^+$  state clearly bears a fundamental similarity to the oxygenation and deoxygenation of erythrocytes. In both, the seat of interaction is intracellular proteins. As far back as 1908, Benjamin Moore pointed out the parallelism of oxygen accumulation in erythrocytes and  $K^+$  accumulation in cells (93). In 1965 I further sharpened this parallelism by pointing out that both oxygen taken up by erythrocytes and  $K^+$  taken up by frog muscle show autocooperative behavior with similar values of nearest neighbor interaction energy (88).

## 7.2 The Control of Cooperative Adsorption and Desorption by Cardinal Adsorbents

Autocooperativity provides the basis for the ability of the solute-adsorbing protein to shift its adsorbed solute from one type to another in a step-wise,

all-or-none manner. According to the AI Hypothesis, such an autocoperative transition is not the property of the protein alone but also of the water molecules, ions, and other substances which interact with the protein molecule at different sites. As a result of the propagated electronic redistribution, all or at least a major share of the properties of the protein-water-ion assembly, are changed. Often, however, it is the physical conformation change that is most noticeable (for a fuller discussion of the various theories of cooperative interaction, see 25). Proteins contain specific sites that exercise a controlling influence over the all-or-none transitions. These sites are called cardinal sites and the specific adsorbents interacting with these cardinal sites are called cardinal adsorbents. ATP is a bona fide cardinal adsorbent, as are many drugs and hormones, and all share a distinctive feature: A small number of cardinal adsorbent molecules can bring about responses involving a much larger number of "regular sites" such as the  $\beta$ - and  $\gamma$ -carboxyl groups adsorbing  $K^+$  and  $Na^+$ .

#### 7.2.1 Ouabain as a Cardinal Adsorbent.

Ouabain, like a number of other cardiacglycosides, causes the loss of  $K^+$  and gain of  $Na^+$  in a variety of living cells. In the conventional membrane-pump model, ouabain acts by inhibiting the Na pump. Indeed it was the parallel behavior of ouabain's effect on the  $K^+/Na^+$  distribution in living cells and its inhibition of the isolated K, Na-activated ATPase that gave impetus to the large amount of work carried out under the assumption that this  $K^+$ -,  $Na^+$ -activated ATPase is in fact that Na pump, and that when incorporated into phospholipid vesicles this pump can actually translocate both K and  $Na^+$  against concentration gradients. Careful analysis of these data revealed major self-inconsistencies in the evidence, and an alternative interpretation of these findings was more in accord with the data (94).

That the K, Na-activated ATPase can indeed adsorb  $K^+$  and  $Na^+$  and that this adsorption is indeed sensitive to ouabain have been demonstrated by Matsui et al as mentioned in the Introduction (31, 32). The ouabain-sensitive

$K^+$  binding on  $K$ , Na-activated ATPase also exhibited autocoooperativity as had been repeatedly demonstrated for  $K^+$  binding in intact living cells.

While Matsui et al's data clearly demonstrated that this enzyme may indeed be the seat of some  $K^+$ ,  $Na^+$ -adsorption in some cells, the demonstration that the A band containing another ATPase, myosin, is the seat of adsorption of the bulk of intracellular  $K^+$  left little doubt that ouabain acts on  $K^+$  and  $Na^+$  distribution in frog muscle and other cells by changing the relative preference of many proteins for the adsorption of  $K^+$  and  $Na^+$ .

Indeed, Figure 2 taken from Ling and Bohr (89) shows that the Yang-Ling cooperative isotherm can quantitatively describe the  $K^+ \rightarrow Na^+$  transition in frog muscle in response to  $3.26 \times 10^{-7}$  M ouabain. Here the full range of external  $K^+/Na^+$  concentration ratios is presented to demonstrate that the primary effect of ouabain is to shift the intrinsic equilibration constant for the  $Na^+ \rightarrow K^+$  exchange in frog muscle from a value from about 100 to a value of 17, i.e., a change by a factor of 6.

Now the normal environment of muscle cells contains 2.5 mM K and 100 mM  $Na^+$ , corresponding to a  $\frac{[K^+]_{ex}}{[Na^+]_{ex}}$  ratio of  $2.5 \times 10^{-2}$ . At this ratio of external  $K^+/Na^+$  concentration, almost all the intracellular anionic sites are occupied by  $K^+$ . The effect of exposure to  $3.26 \times 10^{-7}$  M ouabain is completely to reverse the situation; these sites then become almost entirely occupied by  $Na^+$ .

Ouabain, by its adsorption onto the appropriate cardinal site, alters the selectivity in the adsorption of the cooperatively linked anionic sites causing changes in the c-values in a direction toward a reduced preference for  $K^+$  over  $Na^+$ . Therefore, it is to be expected that if one increases the external  $K^+$  concentration or decreases the  $Na^+$  by a factor equal or greater than the factor of 6, the preferential  $K^+$  accumulation seen in normal cells will be restored. This is of course, true as shown in the data of Figure 2.

### 7.2.2 ATP as a Cardinal Adsorbent.

ATP, for a long time, was considered as carrying special chemical bonds called high energy phosphate bonds. Later work established beyond any question that the enthalpy of hydrolysis of these bonds was not usually high (95) and that the favorable free energy of hydrolysis reflects largely the different affinity its hydrolysis produces for  $H^+$ ,  $Mg^{++}$ , and  $H_2O$  (96, 97). These findings, though little celebrated, contribute to our search for better understanding of this very important chemical compound and its role in physiology. Certainly the old idea that this package of high energy can be made to do work as a sort of universal fuel is no longer tenable.

The AI Hypothesis, by recognizing the role of ATP as a cardinal adsorbent, provides a different mechanism by which ATP energizes biological work performance.

### 8. Maintenance of the Living State: The Role of ATP.

The conventional concept is that hydrolysis of ATP releases energy stored in its "high energy" phosphate bond to support cellular work performance. Thus a resting muscle, on receiving its package of energy from the hydrolyzing ATP, enters into the high-energy contracted state. When ATP is used up, the muscle reverts back to its low-energy relaxed state.

In terms of the AI Hypothesis, quite the opposite is the case. The resting state is seen as a high energy state; its maintenance does not depend on a steady stream of decomposing ATP, but the steady adsorption of intact ATP onto key cardinal sites. Thus the resting state is in a high energy state very much like a set mouse trap. It is only when ATP is destroyed, say by its hydrolysis, that the muscle seeks its low energy state, much as a triggered mousetrap seeks its low energy state.

The simplest and most direct evidence in favor of this basic concept is the fact that a dead muscle is, as a rule, found in the contracted state. Were the relaxed state the lower energy state, dead muscle should be all fully relaxed and rigor mortis would not occur.

Relaxation and shortening are but one aspect of the change in muscle tissue when its ATP content changes. An entirely parallel phenomenon is the inverse relation observed between muscle shortening and desorption of  $K^+$  from the muscle and its release into the medium (19, 24). Perhaps the most convincing and elegant demonstration of the intimate relation between normal muscle contraction and  $K^+$  desorption was that presented by Wilde and coworkers in perfused turtle hearts (98). Each heart beat is accompanied by an exact pulse of labelled  $K^+$  release.

ATP induced changes are not limited to contraction-relaxation, and  $K^+$  desorption-adsorption, important as these changes are, but extend to the physical state of water as well.

#### 8.1 The Control of the Physical State of Cell Water by ATP.

In 1952 I showed that a quantitative relation exists between the  $K^+$  (and  $Na^+$ ) content and the ATP level in frog muscles poisoned with iodoacetate (24). This observation has been repeatedly confirmed and extended (19, 71, 99, 100). There is, generally speaking, also a reciprocal relation between the  $K^+$  and  $Na^+$  contents. This relation can be explained as due to a mechanism similar to that offered for ouabain above. However, the strict one-for-one exchange seen in ouabain induced  $Na^+$  for  $K^+$  exchange was not observed here. It was found instead that there is a parallel between the gain of  $Na^+$  with decreasing ATP and the gain of labelled sucrose (71).

In terms of the AI Hypothesis, the low level of (hydrated)  $Na^+$  as well as sugars and amino acids in normal living cells is due to the polarized-multilayered cell water. Just as ATP acting as a cardinal adsorbent maintains the protein anionic  $\beta$ - and  $\gamma$ -carboxyl groups at a c-value at which  $K^+$  is preferred over  $Na^+$ , so the adsorption of ATP on the same or other proteins maintains the c-value

analogues\* of the backbone carbonyl groups (and the c'-value analogues\* of the backbone imino groups) at values that favor long-range water polarization. This single unitary cause for solute exclusion (in contrast to the multiple and separate causes in terms of membrane pumps) is described as the Universality Rule (66). That is, if for one or another reason, the solubility of one normally excluded solute is changed, then the solubility of all other normally excluded solutes should change pari passu. This rule has been demonstrated in D-arabinose distribution and  $\text{Na}^+$  distribution in frog ovarian eggs (101) and in D-arabinose, sucrose and  $\text{Na}^+$  distribution in the IAA poisoned frog muscles (102). As the muscle or egg cells were dying, their ATP levels gradually fell, and a parallel gain of free  $\text{Na}^+$ , free D-arabinose and free sucrose occurred.

#### 8.2 The Control of Salt Linkage Formation and Dissociation by ATP.

As we have shown, ouabain causes a stoichiometric displacement of  $\text{K}^+$  by  $\text{Na}^+$ . The additional  $\text{Na}^+$  taken up has been shown to be in an adsorbed state as revealed by NMR spectroscopy (103). For a while, it was questioned that the original assumption used in identifying bound  $\text{Na}^+$  might have been erroneous (104). However, it turned out that there was only a quantitative error. The "disappearance" of part of the  $\text{Na}^+$  signal was indeed due to one-site-one-ion specific adsorption of  $\text{Na}^+$ . Thus Monoi's demonstration that the NMR-invisible  $\text{Na}^+$  in liver homogenate can be made visible by the introduction of competing  $\text{K}^+$  and  $\text{Cs}^+$ , but not by choline, indicates ion-specific adsorption (105). If  $\text{Na}^+$  signal disappearance were truly due to a diffuse electric field gradient as suggested by Berendson and Edzes (104), there would be only valency specificity and not the ion specificity which Monoi found. A theoretical argument against the diffuse electric field gradient concept was given by Chang and Woessner (106).

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\* The c'-value is a parameter measuring the positive charge density at a cationic group. c-value analogues and c'-value analogues refer to the negative and positive charge density at polar groups not bearing net charges (19).

The obedience of  $\text{Na}^+$  and sugar uptake in poisoned frog muscle to the Universality Rule, however, shows clearly that the loss of adsorbed  $\text{K}^+$  is largely accompanied by a gain of free  $\text{Na}^+$  (and free sucrose) and is therefore different from ouabain-induced  $\text{K}^+ \rightleftharpoons \text{Na}^+$  exchange.

The question is then raised, "what has happened to the  $\beta$ - and  $\gamma$ -carboxyl groups in the A bands and Z-lines of the muscle that normally adsorb  $\text{K}^+$ ?" According to the AI Hypothesis, in the absence of ATP these anionic sites  $[f^-]$  have disappeared, or become "masked", by forming salt linkages. More specifically, without the ATP these anionic sites  $[f^-]$  prefer as counter ions fixed cations  $[f^+]$  in the form of  $\epsilon$ -amino groups, guanidyl groups,  $\alpha$ -amino groups and/or histidyl groups thus forming salt linkages  $f^+f^-$  (53, 19, 107).



We are uncertain about the nature of the counteranion ( $\text{X}^-$ ) adsorbed to the fixed cation before the salt linkage formation: in muscle tissues it could be creatine phosphate.

If salt linkages are formed among different protein molecules, there may be macroscopic volume shrinkage and loss of water with a concomitant change of the state of water. On the other hand, if the salt linkages are formed within a single protein molecule or an aggregate of protein molecules, the result might be limited to the conformation change of that protein.

### 8.3 Synchronized ATP- and ATPase-dependent Cyclic Changes.

Addition of  $\text{Mg}^{++}$  and ATP to a suspension of paramacia that had been glycerinated evoked synchronized beating of the cilia propelling the dead protozoas in water as if they were alive (108). An interpretation of this remarkable phenomenon on the basis of the AI Hypothesis was offered (21). In brief, ATP acts as a

cardinal adsorbent causing cilia orientation in one way followed by  $Mg^{++}$ -induced ATP hydrolysis and the resultant protein conformation change causing cilia orientation in another direction. The beating cycle is then re-initiated by adsorption of fresh ATP. This model clearly establishes the need for a continuous supply of fresh ATP in order to promote continued swimming.

Another equally fascinating illustration of the inherent ability of protoplasm to act in a synchronized and cyclic manner is the ATPase-dependent ion accumulation and swelling cycles of rat liver and pigeon heart mitochondria, the latter initiated by valinomycin and monactin (109, 110).

The evidence that valinomycin, monactin, etc. do not act as ionophores but rather as cardinal adsorbents has been discussed recently (25). It only needs to be mentioned here that  $Sr^{++}$ , a simple divalent ion with no ionophore property whatsoever can initiate cyclic changes in much the same way as valinomycin in other studies (111).

The oscillatory changes are in perfect synchrony in regard to shrinkage and swelling with concurrent loss and gain of  $K^+$ , and they involve the operation of an ATPase. It is my belief that this basic protoplasmic trait is essential to a variety of physiological activities including transepithelial transport of water and ions.

#### 9. Cooperative Adsorption-desorption Model of Active Transport Across Epithelia and Other Bifacial Cell Systems.

When living cells are incubated in a  $K^+$ -free or low  $K^+$  solution, they gain  $Na^+$  and lose  $K^+$  until a new equilibrium is reached. In the case of isolated frog muscle, a tenfold reduction in the external  $K^+$  concentration from 25 to 0.25 mM at constant external  $Na^+$  concentration (100 mM) leads to almost total displacement of  $K^+$  by  $Na^+$  (see Figure 2). If these  $K^+$ -depleted muscles are returned to a normal Ringer solution, their  $Na^+$  will be stoichiometrically displaced by  $K^+$ . In

this restoration process both  $\text{Na}^+$  and  $\text{K}^+$  are "transported" against concentration gradients. This type of phenomenon has been referred to as active transport by adherents of the membrane-pump theory. However, now that we have established that the  $\text{K}^+$  in the cells is virtually all adsorbed (Figure 1) and so is the  $\text{Na}^+$  that stoichiometrically replaces  $\text{K}^+$  (112), the phenomenon can no longer be regarded as active transport. Indeed the phenomenon represents truly an exchange adsorption much as we witness in the operation of an ion exchange resin. The displacement of  $\text{K}^+$  by  $\text{Na}^+$  adsorbed on Dowex 50 is not an active transport process but merely the consequence of a change in the ratio of  $\text{K}^+/\text{Na}^+$  concentration in the external medium and the fact that electrostatic and other forces make it more favorable for one ion to congregate in the resin than an alternative ion at the ambient concentration ratio. Oxygen is concentrated in red blood cells for exactly the same reason.

If a hemoglobin solution kept inside a dialysis bag is suspended in an oxygen-containing solution, after a suitable length of time an equilibrium will be reached and the amount of oxygen taken up by the sac is quantitatively defined. If now ATP is added to the internal solution, oxygen will begin to move out of the hemoglobin-containing phase into the external solute (71, 113). This outward migration of oxygen is the consequence of ATP interaction with the oxygen-binding protein, hemoglobin. ATP exercises a long-range effect on the affinity of the heme groups for oxygen, a type of effect called "allosteric" by Monod et al (114) (A detailed mechanism for such allosteric effect has been offered earlier in the AI Hypothesis and was referred to as an indirect F-effect (20, 21)). When ATP is removed from the system, oxygen will once more move into the bag, apparently against a concentration gradient. This effect of ATP on the concentration of a solute in the hemoglobin-containing system does not involve the hydrolysis of ATP, since hemoglobin has no ATPase activity.

This movement of the solute oxygen into and out of the hemoglobin-containi

system involves the participation of the laboratory worker. Without his or her effort, it would be impossible to introduce or remove ATP from the system and hence to bring about the transport of oxygen. One may imagine another way to achieve the cyclic back and forth transport of oxygen, if the bag also contains first a specific enzyme, an ATPase, which can destroy and thus remove ATP from the hemoglobin-containing phase; second, another enzyme system that can regenerate ATP; and third, a coordinating system that can synchronize the ATP destruction and its subsequent resynthesis and readsorption. That biological systems are so designed to provide just this kind of synchrony and coordination, has already been shown in the example of ATPase-dependent swimming of dead paramecia and ATPase-dependent oscillatory changes of ion and water uptake and release in isolated mitochondria suspensions.

In summary, through the use of simple models, we have demonstrated that it is possible to understand how a cyclic change of ATP adsorption and hydrolysis can bring about a cyclic change of selective accumulation of a solute from the medium and its subsequent release back into the medium. I shall now try to incorporate this mechanism into a theoretical model that can perform true active transport. The major additional component we need is a one way-valve.

As we discussed above, the surface permeability barrier of the living cell is not a lipid layer but multilayers of water polarized by cell surface proteins. It is then reasonable that the synchronized cyclic ion and water release and uptake involves an alternation between a polarized impermeable state and a depolarized, permeable state of the surface water. If we can imagine that the basolateral or serosal surface has these properties and that the apical or mucosal surface does not undergo cyclic changes but has high c-value anionic sites and hence selective high permeability for  $\text{Na}^+$ , we will then be equipped with the necessary components for active transport.

#### 9.1 A Detailed Model of Active Transport of Solutes and Water.

Figure 3 outlines the model of active transport in 4 stages. The ion to

be transported from the external solution,  $\text{Na}^+$ , is represented as solid triangles. The mucosal surface is considered as having a higher permeability for  $\text{Na}^+$  than the resting serosal surface because the serosal surface anionic sites have a high c-value that promotes selective  $\text{Na}^+$  adsorption. Water in the serosal surface, the cytoplasm as well as the normal cell surface exists in the polarized multilayered state at the beginning of the cycle when the cell is at rest. In this active transport model the key role is played by the cytoplasmic and serosal surface proteins which possess an ATP binding cardinal site as well as an ATPase activated by the transported ion. It is suggested that the ATP-binding cardinal site is in fact the ATPase site but in a different cooperative state.

Stage 1. At this stage the higher mucosal surface permeability allows both  $\text{Na}^+$  and water to enter into the cell. Once inside, the  $\text{Na}^+$  and water will proceed to adsorb onto anionic sites on the cytoplasmic proteins which are under the control of the cardinal adsorbent, ATP, and thus exist in an extended state with their backbones favoring multilayer polarization of water and their anionic side chains preferring  $\text{Na}^+$ .

The question may be raised, "How can we reconcile this stipulation with the view that ATP adsorption favors  $\text{K}^+$  (rather than  $\text{Na}^+$ ) adsorption on cell proteins?" The answer lies in the fact that the ATP control of the c-value ensemble depends on other factors, e.g., secondary cardinal adsorbents. ATP adsorption favors  $\text{K}^+$  adsorption is specific only for a specific protein under rigorously defined conditions. It also bears remembering that in active transport across bifacial cells the key cation is not always  $\text{Na}^+$ . Thus in Malpighian tubules of insects the key ion transported is  $\text{K}^+$  (117). This stochastic process continues until the protein enters into the cooperative  $\text{Na}^+$  state with a high concentration of adsorbed  $\text{Na}^+$  locally accumulated.

Stage 2. The autocoperative shift to the  $\text{Na}^+$  state involves the site adjacent to the cardinal site which also adsorbs  $\text{Na}^+$ . This  $\text{Na}^+$  adsorption then activates the Na-K-activated ATPase activity of the cardinal site, causing the

hydrolysis of ATP, thereby entering Stage 2.

With the hydrolysis of ATP, the protein undergoes an autocoooperative desorption of  $\text{Na}^+$  (possibly of  $\text{Cl}^-$  as well) with the formation of salt linkages, depolarization and release of water and the liberation of a high concentration of  $\text{Na}^+$ . At the same time or slightly later the serosal surface protein also undergoes change from the extended to a more helical conformation with water depolarization.

Stage 3. In the third stage, depolarization of serosal surface water increases the serosal surface permeability to ions and osmotic flow of water permitting rapid exit through the serosal surface of both the liberated  $\text{Na}^+$  and released water.

Stage 4. This last stage is marked by the regeneration of ATP, its adsorption onto the cardinal site, and autocoooperative shift back to the Stage 1 condition: favoring  $\text{Na}^+$  adsorption on anionic side chains and multilayer polarization at the serosal membrane. The cycle is now ready to repeat itself.

## 9.2 Discussion of the Current Model.

### 9.2.1 Cyclic Changes of Adsorption-desorption as the Basis of Active Transport.

The cyclic changes of adsorption-desorption proposed in this model may not be easily observable in a multicellular epithelium. However, there is evidence of cyclic changes in the case of single giant algal cells largely through the careful work of S. C. Brooks (115, 116). Thus when *Nitella* cells were exposed to salt solutions containing radioactive  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Rb}^+$ , these ions first became accumulated in the protoplasmic layer surrounding the central vacuole. Furthermore, the accumulation of these labelled ions was not monotonic but exhibit a distinct periodic increase and decrease, and they reached a concentration in the protoplasm many times higher than in the surrounding medium. It is considerably

later that the labelled cation reaches the cell sap in the central vacuole. Figure 4, taken from Brooks (115), shows the time course of labelled  $Rb^+$  accumulation in the sap. The rise phase of sap  $Rb^+$  coincides with the fall phase of the protoplasmic  $Rb^+$ ; while the fall phase coincides with the rise phase of the protoplasmic  $Rb^+$ . Eventually the sap  $Rb^+$  reached concentrations "notably exceeding those present in the immersion fluid." At no time was the concentration in the sap higher than that in the protoplasm.

#### 9.2.2 Location of the Pumping Mechanism Relative to Ussing's Two Membrane Model.

Our present model has incorporated the two-membrane theory of Ussing to the extent of recognizing and utilizing the different permeability characteristics of the serosal and mucosal membrane as first discovered by Koefoed-Johnson and Ussing (9). Otherwise, the present model, based on the general thesis of the AI Hypothesis, rests on a different foundation than the conventional membrane-pump theory. In the detailed model, we have presented the part of the protoplasm involved in the adsorption-desorption cycle to include the entire cytoplasm as well as the serosal surface, because there is experimental evidence suggesting the involvement of the entire cell content (see below). However, on purely theoretical grounds, the protoplasm involved in the cyclic changes may be entirely limited to the serosal surface. Under this condition it would approximate the location of pumping proposed by Ussing's two membrane theory.

The evidence that the cyclic pumping mechanism is not confined exclusively to the serosal cell surface includes the following: (1) Maddrell showed that the rate of transport of  $K^+$  and  $Na^+$  by the isolated Malpighian tubule of the blood sucking insect, *Rhodnius*, is strongly correlated with the respective total intracellular concentration of each of these ions (117). (2) Spring and Giebisch showed that the rate of net Na transport in perfused *Necturus* kidney cells was linearly related to the total intracellular  $Na^+$  concentration (118). Again no

such linear correlation could be expected if the bulk of cell  $\text{Na}^+$ , a substantial portion of which is adsorbed (52), is not involved in the transport. (3) Morel showed that after injection of  $\text{K}^+$  into rabbits the specific activity of urinary  $\text{K}^+$  quickly attains that of the renal tissues but does not follow the time course of specific activity in the arterial plasma (119). The results support the view that it is  $\text{K}^+$  that has undergone effective exchange with cell  $\text{K}^+$  that plays a major role in urinary  $\text{K}^+$  excretion. Again this suggests the involvement of more than a small amount of adsorbed  $\text{K}^+$  on the serosal surface in the process (see Table 1).

#### 9.2.3 The Source of Energy for the Active Transport.

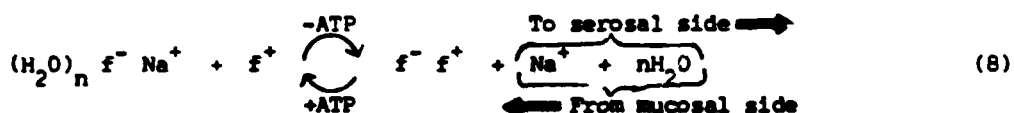
In the present model, the immediate source of energy is stored in the protein-water-ion assembly in the high energy resting state, and the ultimate source of energy for transport of solutes and water is that used in the synthesis of ATP from ADP and  $p_i$ . If one assumes that this synthesis is highly effective, with an efficiency of 100%, then the energy formed would be proportional to the free energy liberated during ATP hydrolysis. Further, since there is a fixed number of ATP molecules synthesized for each molecule of oxygen utilized, the energy for active transport should also be quantitatively related to the extra oxygen consumed.

Recently Ussing and Leaf raised an old question, "whether there is a stoichiometric relationship between the number of ions transported and the amount, of say, ATP consumed..." (7, p. 3). They cited earlier work of their own laboratory and others leading to the general conclusion that there may be a correlation between  $\text{Na}^+$  transport and oxygen consumption of, for example, frog skin. On the other hand, the failure to demonstrate such a relation in all tissues may reflect either a predominantly glycolytic source of ATP regeneration, or the presence of a high and variable rate of oxygen consumption for cell functions not directly concerned with active transport.

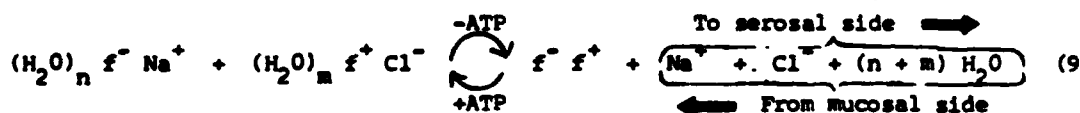
#### 9.2.4 Coupling of Ion and Water Transport.

Gall bladder, proximal kidney tubules, and small intestine are examples of "low resistance" epithelia. They transport water in the form of an isotonic fluid. As mentioned above, the standing gradient osmotic flow theory was proposed by Diamond and Bossert (10). This theory, a refinement of the earlier double membrane theory of local osmosis to explain the apparent coupling of solute and water flow (120, 121), showed that contrary to earlier assumptions a second membrane is not necessary and that any confined space lined with a membrane possessing both active salt transport and semipermeability could function as a local osmotic coupling space. A. E. Hill pointed out that the feasibility of this theory critically depends on the osmotic permeability of the membrane involved and that the required permeability range of  $10^{-1}$  to  $10^1$  cm<sup>2</sup>/s "lies completely outside that of any living... membrane studied to date by at least three orders of magnitude" (122).

The present adsorption-desorption model may be represented in the case of NaCl coupled water transport by the two sets of alternative equations:



or



As shown in Figure 3, the synchronized dephosphorylation of ATP at Stage 2 causes simultaneous liberation of Na<sup>+</sup> from its adsorption on anionic sites f<sup>-</sup>, and of water from its polarized multilayered adsorption on the extended polypeptide chain NHCO sites. A normal free aqueous solution of water and Na<sup>+</sup> and

$\text{Cl}^-$  is momentarily and locally released concomitant with the removal of the barrier to ion permeation as well as to osmotic water flow at the serosal cell surface. This is so because cooperative multilayer polarization of water drastically reduces osmotic permeability but only moderately reduces diffusive permeability while depolarization of water increases both. As a result an essentially isotonic fluid of  $\text{Na}^+$  is excreted into the lumen of the "low resistance" epithelia.

It may be mentioned that solute and water transport may be rate-limited by either the frequency of the cycles of adsorption and desorption or by the mucosal surface permeability. The fact that antidiuretic hormones when applied to the outside surface of frog skin and toad bladder greatly increase the rate of  $\text{Na}^+$  transport as well as osmotic water permeability without major effect on diffusional permeability (123) suggests that this hormone may depolarize water at the mucosal surface thereby increasing both  $\text{Na}^+$  and water permeability at the external or mucosal surface.

While the cyclic adsorption-desorption model given above can explain active coupled salt and water transport with only the serosal surface involved in polarization-depolarization cycling of permeability changes, there is no reason why in different tissues the cycling may not occur at the mucosal rather than the serosal surface, as is known or at least indicated in insect Malpighian tubules, insect midgut, choroid plexus and gastric mucosa.

#### 9.2.5 Conciliation of the Conflict Between Models of "Homocellular Regulation of Cell $\text{K}^+$ and $\text{Na}^+$ Composition" and "Homoepithelial $\text{Na}^+$ Transport".

Schultz wrote in a recent review on "Transport Across Small Intestine": "The data available at present cannot be readily reconciled with any model that invokes a close relation between transepithelial  $\text{Na}^+$  transport and the homocellular regulation of  $\text{Na}^+$  and  $\text{K}^+$  composition or with the notion that the epithelial cell can be adequately represented by a double membrane model" (124, p. 769).

These limitations and conflicts are primarily the consequence of the incorrect basic concepts of the membrane-pump theory of the living cells.

The present model does not encounter any internal conflict between a theory of the mechanism of selective  $K^+$  accumulation and  $Na^+$  exclusion in epithelial cells (and algal cells) and the theory of transepithelial  $Na^+$  transport. Indeed, the transepithelial  $Na^+$  transport model is built on the foundation of the mechanism for "homocellular"  $K^+$  accumulation and  $Na^+$  exclusion.

#### 9.2.6 Coupling of $Na^+$ Transport with Sugar and Amino Acid Transport.

An outstanding event in the modern history of transepithelial solute transport was the discovery of cotransport of  $Na^+$  and sugar and the cotransport of  $Na^+$  and amino acids across epithelial systems (12, 13, 14). However, the interpretation thus far offered, the Na gradient concept, is based on the assumption that cell water is free. Since this assumption is now disproven, the subsidiary assumption that an electrochemical gradient of  $Na^+$  exists between the mucosal fluid and the cytoplasm is also disproven. I shall now very briefly present a recasting of these important findings in terms of the cyclic adsorption-desorption model of transepithelial transport.

##### 9.2.6.1 Surface Protein Adsorption Sites to Replace the "Membrane Carriers".

In the conventional view, the mutual dependency of  $Na^+$  and sugar (or amino acid) in their rate of transepithelial transport is due to the mandatory requirement of the formation of a ternary complex of  $Na^+$ -carrier-sugar for the permeation into the epithelial cells (15, 18). The carrier coupled either to  $Na^+$  or to sugar alone is not able to permeate into the cell or at least permeates very much more slowly. The fact that the cell membrane barrier is now known not to be due to phospholipid demands a reconstruction of this theoretical model.

As discussed earlier, polarized water offers strong

resistance to permeation of sugar or  $\text{Na}^+$ . To enter the cells, these solutes must take the adsorption-desorption route via surface protein sites. Under normal conditions these cell surface proteins offer few or no specific adsorption sites for  $\text{Na}^+$  or D-glucose. However, in the presence of both  $\text{Na}^+$  and D-glucose, the surface proteins undergo a cooperative transformation to assume a new conformation with a new c-value profile. In this new conformation, the surface protein offers both anionic sites of the proper c-value for  $\text{Na}^+$  adsorption and a combination of sterically suitable backbone and side chain sites to adsorb D-glucose specifically. The subsequent entry will follow typically the adsorption-desorption route earlier described for simple ion entry. Since D-glucose and  $\text{Na}^+$  adsorption is cooperative, desorption of D-glucose or  $\text{Na}^+$  will facilitate the desorption of  $\text{Na}^+$  and D-glucose respectively, hence the enhanced simultaneous entry of  $\text{Na}^+$  and D-glucose.

#### 9.2.6.2 Studies of Isolated Microvilli from Intestinal and Kidney Epithelia.

In recent years techniques for the isolation of microvilli from intestinal mucosa and from rat or rabbit epithelia have been developed and steadily improved (125). Typically the rate of D-glucose (but not L-glucose) or amino acid uptake is greatly accelerated if the external medium contains a high concentration of  $\text{Na}^+$  and the microvilli contain a low concentration of  $\text{Na}^+$ . Preincubation of the microvilli with a medium containing a high  $\text{Na}^+$  concentration, the inclusion of phoridzin in the incubation medium or the substitution of  $\text{K}^+$  for  $\text{Na}^+$  in the external medium all slow down the initial uptake. However, if valinomycin is added to a K-preloaded vesicle, the D-glucose uptake is again accelerated. Similarly the addition of the uncoupler, CF-CCP in proton-loaded microvilli also produces an acceleration of the initial D-glucose uptake rate. The conventional interpretation was that D-glucose uptake was driven by the  $\text{Na}^+$  gradient, or in the presence of valinomycin, the  $\text{K}^+$  gradient or in the presence of CF-CCP, a proton gradient.

9.2.5.2.a The Equilibrium Level of D-glucose.

The accelerated uptake described usually appears in the form of an "overshoot". That is to say, that the sucrose quickly reaches a peak in the microvilli and then declines until a much lower and steady level is reached. It would seem that the relative height of this level can give us some additional insight into the mechanism which maintains or controls the level of D-glucose, and other solutes in living cells.

It is generally agreed that in the intestinal as well as kidney tubule epithelium the postulated Na pump is located at the serosal surface and that there is no outward  $\text{Na}^+$  pump on the mucosal microvilli surface. The demonstrated osmotic activity of the isolated microvilli shows that most of the microvilli are resealed at the broken end. Thus the isolated microvilli present a unique system that contains no outward Na pump that has been postulated universally for all living cells. Following the logic of the conventional membrane-pump theory, one would expect that the steady level of sugar reached in the microvilli must be equal to that in the bathing solution.

In Table 2 I have calculated from the data in the literature two sets of apparent equilibrium distribution coefficients or  $\rho$  values for D-glucose, L-alanine and L-lactic acid. The apparent distribution coefficient at the peak of the "overshoot" is expressed as  $\rho_{\text{max}}$ . The apparent equilibrium distribution coefficient for each of these solutes at the final equilibrium level is represented as  $\rho_{\text{eq}}$ . The data given here calculated on the assumption that the external concentration of D-glucose, etc., did not significantly differ from that given as the initial concentration of the medium and that the microvilli contain 80% water. Table 2 shows that while  $\rho_{\text{max}}$  occasionally rose above unity,  $\rho_{\text{eq}}$ , without exception, is below unity and is as a rule more or less at the same level as in intact cells. These data contradict the membrane-pump theory but are in full

accord with the AI Hypothesis: Water in the cell cytoplasm in the microvilli, as well as elsewhere, exists in the state of polarized multilayers and as such has reduced solubility for all solutes, including D-glucose, L-glucose, L-lactate, and L-alanine.

#### 9.2.6.2.b The Overshoot.

I have already suggested that the synergistic adsorption of  $\text{Na}^+$  and D-glucose onto surface protein sites facilitates the entry of  $\text{Na}^+$  and D-glucose into the microvilli. I now suggest that the "overshoot" represents a transient adsorption of D-glucose onto some cytoplasmic proteins. In support of this concept, are the following observations: (i) As shown in Table 2, the apparent maximum  $\theta$ -value achieved at the peak of the overshoot often exceeds unity. In terms of the AI Hypothesis, an above unity  $\theta$ -value as a rule is due to selective adsorption or other form of complex formation in the cell. (ii) No overshoot is discernible in liposomes incorporating proteins extracted from microvilli (126, 127). In these cases, the vesicles are hollow rather than solid as in the isolated microvilli; the cytoplasmic proteins essential for the transient D-glucose, L-alanine adsorption are of course not present. (iii) The strong dependence of the overshoot on anions known for their strong adsorption on anionic sites (e.g.,  $\text{SCN}^-$  vs.  $\text{Cl}^-$ ) (see 19, p. 172 for compiled data on the relative adsorption energy of the anions on proteins in general) suggests that the adsorption of D-glucose involves unmasking of sites when  $\text{Na}^+$  and  $\text{SCN}^-$  join in dissociating salt linkages as in Equations 8 and 9 in the direction from right to left. (iv) We have already mentioned that the cell surface barrier is not primarily lipid in nature and that valinomycin and monactin could not function as ionophores (for detailed discussion, see 25). Rather, they function as cardinal adsorbents, controlling the electronic as well as steric conformation of certain specific proteins, affecting  $\text{K}^+$  adsorption. Similarly, CP-CCP acts as a cardinal adsorbent to bring anionic sites to a c-value affecting  $\text{H}^+$  binding (i.e., by increasing the c-value).

However, the question why the increased adsorption does not sustain itself but declines is less amenable to explanation. Does it correspond to part of the cyclic change proposed? Or is it only a transient change of a deteriorating system? All these and many other questions can only be answered by future studies.

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### Legends

Figure 1. Electron micrograph of dry cut, unstained section of freeze-dried frog sartorius muscle. A EM of muscle fixed and stained with uranium-lead by conventional procedure. B EM of  $\text{Cs}^+$ -loaded muscle, without chemical fixation or staining. C  $\text{Tl}^+$ -loaded muscle without chemical fixation or staining. D Same as C after exposure of section to moist air, which creates granular  $\text{Tl}^+$  deposits from its hitherto even distribution in the A band. E Section of central portion of B after loading in distilled water. F Normal " $\text{K}^+$ -loaded" muscle. (A Partial reproduction of EM from H. Huxley (143), B to F from L. Edelmann (56))

Figure 2. Effect of Ouabain ( $3.2 \times 10^{-7}$  M) on the Equilibrium Distribution of  $\text{K}^+$  and  $\text{Na}^+$  Ion. Curves with open ( $\text{Na}^+$ ) and filled ( $\text{K}^+$ ) circles were equilibrium distribution data from muscles not treated with ouabain. The point of intersection gives a  $K_{\text{Na} \rightarrow \text{K}}^{\text{oo}}$  of 100. In muscles treated with ouabain ( $3.2 \times 10^{-7}$  M),  $K_{\text{Na} \rightarrow \text{K}}^{\text{oo}}$  has shifted to 21.7.

Figure 3. Cyclic adsorption-desorption model of active transport of Na ( $\Delta$ ,  $\Delta$ ) across frog skin, intestinal epithelia and other bifacial systems.

Figure 4. The concentration of  $\text{Rb}^+$  in the protoplasm and in the sap of *Nitella* internodal cells during 120 hours of immersion in 0.005 M  $\text{Rb}^+\text{Cl}$  (means of five series). pH = 7.3; continuous illumination contains 0.0001 M  $\text{CaCl}_2$  (experiment Rb 10). (S.C. Brooks, J. of Cell Comp. Physiol. 14: 383 (1939) - by permission)

Table 1 Collected data of the  $\text{K}^+$  concentration ( $c_K^i$ ),  $\text{K}^+$  activity ( $a_K^i$ ) and activity coefficient ( $a_K^i/c_K^i$ ) in varying epithelial cells measured with intracellular  $\text{K}^+$ -sensitive electrodes.

Table 2 The apparent maximum equilibrium distribution coefficient for D-glucose, L-glucose, L-lactate and L-alanine in isolated microvilli from intestinal

and renal epithelia.  $P_{\max}$  is the apparent distribution at the peak of the overshoot and  $P_{eq}$  reflects the final equilibrium value.

Ling

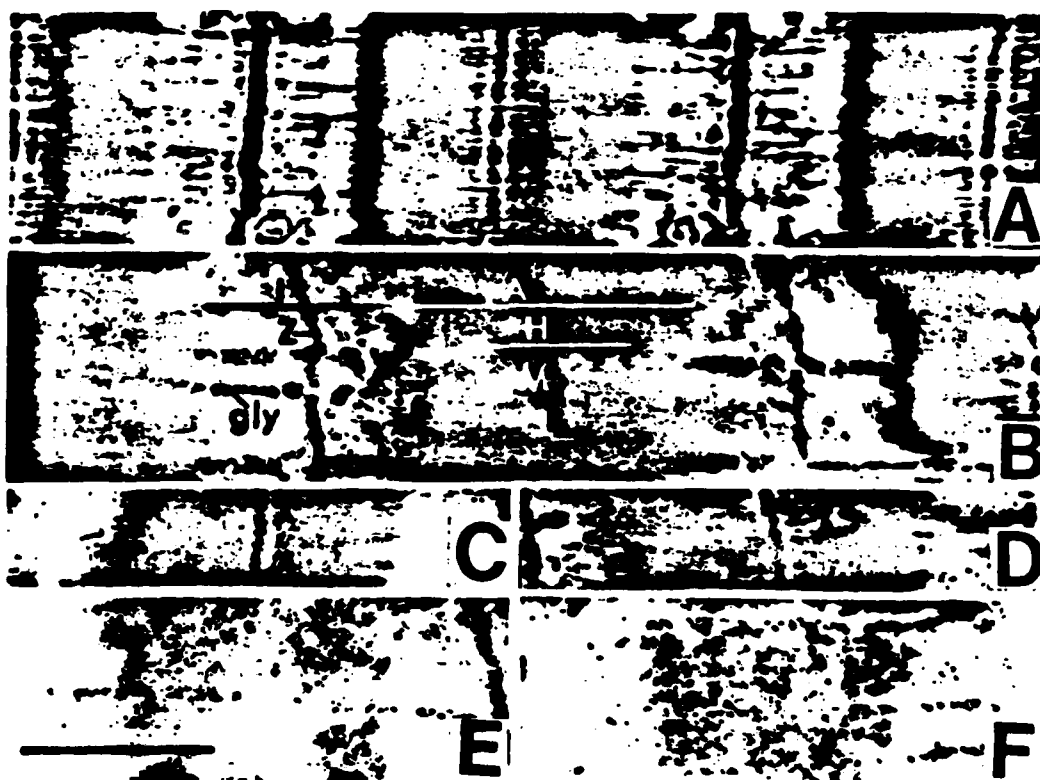


FIGURE 1

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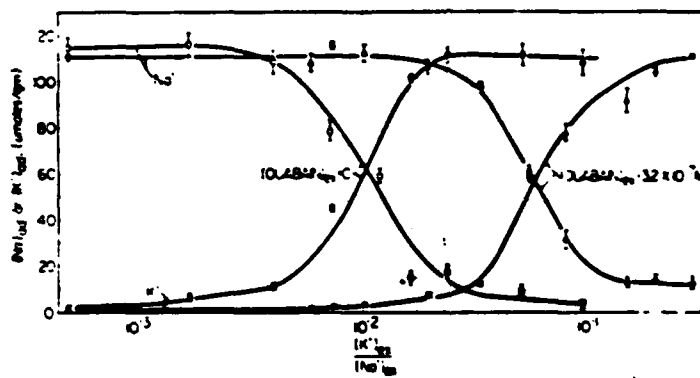


FIGURE 2

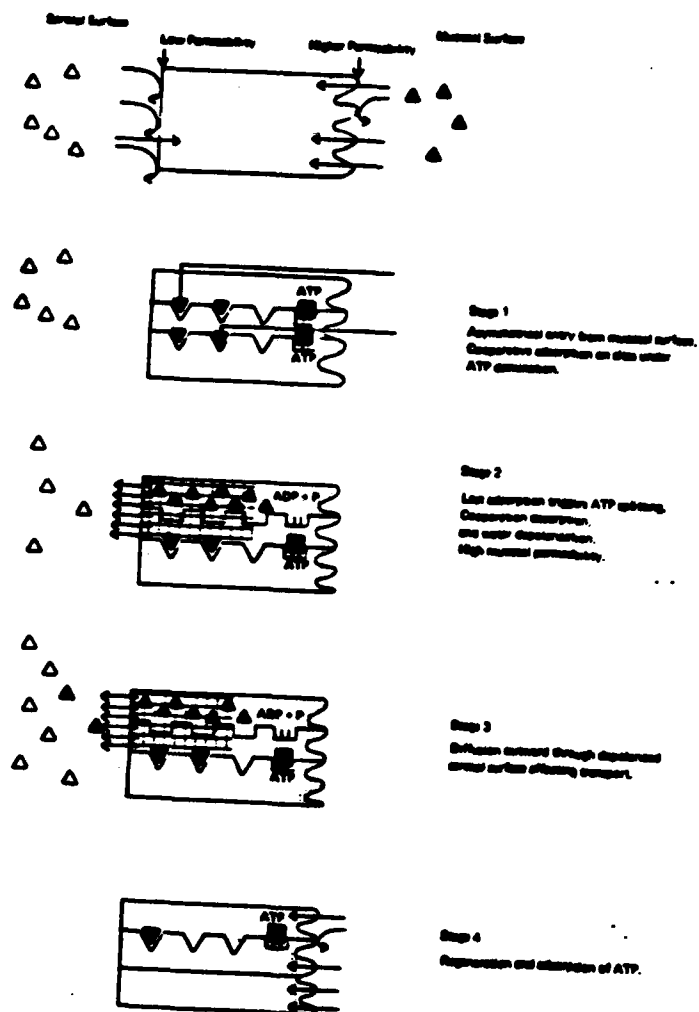


FIGURE 3

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